Assay Methods for Zearalenone and its Natural Occurrence

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Since earlier reports on the structure, chemical, and biological properties of zearalenone (F-2) (Stob *et al.,* 1962; Christensen *et al.,* 1965; Mirocha *et al.,* 1967), there have been numerous reports of its natural occurrence in agricultural commodities. Some of the commodities were analyzed specifically for zearalenone because they had been implicated in field outbreaks of mycotoxicosis in farm animals. Other grains were tested because of obvious mold damage. Surveys have also been made on grains moving through commercial channels and on farms and at country elevators to determine the incidence of zearalenone to be expected in grains at different points in the marketing system and from various geographical regions. Reviews by Erikson (1968), Hesseltine (1974), and Stoloff (1976) on natural occurrence of mycotoxins have included information on zearalenone.

Zearalenone has been detected in hay, feed, corn, pig feed, sorghum, dairy rations, and barley that had caused field problems in farm animals in England, Finland, Yugoslavia, Scotland, and United States (Table 1). Mirocha *et al.* (1974) reported the natural occurrence of zearalenone in a number of feeds and grains that had been involved in mycotoxicosis. Most of the problems reported were caused because of its estrogenic properties and included infertility in cattle and swine, and stillbirths, neonatal mortality, and small litters in swine. Zearalenone was also detected in corn and grain sorghum that had obvious mold damage (Table 1). It was detected in freshly harvested corn (Caldwell and Tuite, 1974) as well as in corn that had been stored in a bin for several months (Shotwell *et al.,* 1975). Zearalenone was detected in samples taken from several parts of the bin, but at the time of detection there was no way of determining when zearalenone had been formed.

Surveys of corn in the United States for zearalenone have been reported by the Northern Regional Research Center and the Food and Drug Administration (Table 2). Corn samples moving in commercial channels were collected by Grain Inspection, Agricultural Marketing Service, for NRRC in the

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Table 1: Natural Occurrence of Zearalenone

Commodity or product	Examined because of:	Country	Zearalenone (ppm)	Reference
Hay	Infertility in dairy cattle	England	14.0	Mirocha *et al.*, 1968
Feed	Infertility in cattle and swine	Finland	25.0	Korpinen, 1972
Corn	Hyperestrogenism in farm animals	France	2.3	Jemmali, 1973
Animal feed	Hyperestrogenism in cattle and swine	United States	0.1–2,900	Mirocha *et al.*, 1972
Corn		Yugoslavia	18	Hesseltine, 1974
Corn	Poisoning in swine	Yugoslavia	2.5–35.6	Osegovic, 1970
Corn	Severe mold damage and swine refusal	Yugoslavia	0.7–14.5	Nowar, 1975
Barley	Stillbirths, neonatal mortality, and small litters in swine	Scotland	0.5–0.75	Miller *et al.*, 1973
Corn (freshly harvested)	*Gibberella zeae* damage	United States	0.1–1.5	Caldwell and Tuite, 1974
Corn (stored)	*A. flavus* damage	United States	ND-92[1]	Shotwell *et al.*, 1975
Barley	Death in swine	Scotland	"traces"	Shreeve *et al.*, 1975
Feed	Field problems in animals	United States	Not stated	Meerdink and Buck, 1975
Grain sorghum	Head blight in sorghum	United States	Not stated	Schroeder and Hein, 1975
Corn	Swine hyperestrogenism	Yugoslavia	35.6	Mirocha *et al.*, 1974
Pig feed	Swine hyperestrogenism	United States	50.0	Mirocha *et al.*, 1974
Corn	Swine hyperestrogenism	United States	2.7	Mirocha *et al.*, 1974
Sorghum	Cattle abortion	United States	12.0	Mirocha *et al.*, 1974

(*continued on next page*)

Table 1 (*Continued*)

Commodity or product	Examined because of:	Country	Zearalenone (ppm)	Reference
Corn	Swine abortion	United States	32.0	Mirocha *et al.*, 1974
Silage		United States	87.3	Mirocha *et al.*, 1974
Corn		England	306.0	Mirocha *et al.*, 1974
Corn	Swine feed refusal	United States	2.5	Mirocha *et al.*, 1974
Dairy ration	Cattle feed refusal, lethargy, anemia	United States	1.0	Mirocha *et al.*, 1974
Pig feed	Swine internal hemorrhaging	United States	0.1	Mirocha *et al.*, 1974
Pig feed	Swine hyperestrogenism	Yugoslavia	0.5	Mirocha *et al.*, 1974
Pig feed	Swine infertility and abortion	United States	0.01	Mirocha *et al.*, 1974

[1] Samples taken from several parts of the bin rather than blending entire lot.

corn belt (Shotwell *et al.*, 1970) and from export cargos (Shotwell *et al.*, 1971). The samples collected by grain inspectors, AMS, included corn of all grades. The two zearalenone-positive samples collected in the corn belt were in the poorest grade—Sample Grade. The positive samples collected from export cargos were in Sample Grade, U.S. No. 4, and U.S. No. 3. The incidence (1–2%) and levels (0.4–0.8 ppm) of zearalenone in the corn were low.

The incidence (17%) and levels (0.4–5.0) of zearalenone were understandably higher in an FDA survey of 1972 corn conducted by Eppley *et al.* (1974) in the spring of 1973. Samples were collected in an area where known Fusaria damage had been reported or where the potential for Fusaria damage was considered to be higher. During the 1972 growing season, the corn belt had experienced unusually wet weather that delayed planting in the spring and harvesting in the fall. Some corn was not harvested until January. There were numerous reports of Fusaria infection in the area, and a high incidence of zearalenone as well as of other Fusaria toxins was expected.

A survey of 1973 corn collected from different regions of the United States (Stoloff *et al.*, 1976) revealed that the corn belt with a 10% incidence experienced more problems with zearalenone contamination than other areas (1% incidence). Levels of zearalenone encountered in any of the 1973 corn samples were low (<0.4 ppm). The samples of marketable corn had been collected at farms and country elevators.

Mirocha *et al.* (1972) reported a survey for zearalenone in 139 samples of corn to be used for human consumption in Mexico (Table 2). The incidence of toxin was 4%, but levels were not mentioned.

Wheat and soybean samples collected by AMS from the southeast and western part of the corn belt were analyzed for zearalenone, ochratoxin, and aflatoxin to determine their susceptibility to formation of these mycotoxins (Shotwell *et al.*, in press). None of the three mycotoxins were detected in the 180 soybean samples analyzed. The wheat samples did not have detectable aflatoxin or ochratoxin by the method used (Eppley, 1968). However, 19 of the 54 wheat samples collected in the southeast had levels of zearalenone from 0.4 to 11.0 ppm (Table 3). Many of the 54 samples had been collected because they were obviously mold damaged. Results obtained by thin-layer chromatography (TLC) were confirmed by gas liquid chromatography (GLC) and mass spectroscopy (MS). Fusaria were isolated from zearalenone-positive samples. The 58 samples collected from the western part of the corn belt were negative. If there were a 5% incidence of zearalenone in samples from the corn belt, there would be a 95% probability that one positive sample would be found.

Aflatoxin has been detected—usually at lower levels—in corn containing zearalenone (Table 4). Both zearalenone and T-2 toxin were reported to be present in a sample of mold-damaged barley. One lot of 1972 crop corn from Yugoslavia that was implicated in swine refusal and lowered weight gains in cattle and swine contained 3.1 ppm ochratoxin and 14.5 ppm zearalenone.

Table 2: Surveys of Corn for Zearalenone

Year	Agency surveying	Origin samples	Type sample and source	Number of samples assayed	Per cent samples with indicated level of zearalenone (ppm)				Reference
					ND[1]	<0.4	0.4–0.9	1.0–5.0	
1967	NRRC	Corn belt	Grain inspection AMS	283	99		1		Shotwell *et al.*, 1970
1968–1969	NRRC	Export cargo	Grain inspection AMS	293	98		2		Shotwell *et al.*, 1971
1972	FDA	Corn belt[2]	Elevator and food processing	223	83	9	4	4	Eppley *et al.*, 1974
1973	FDA	Corn belt	Farm and country elevator	169	90	10			Stoloff *et al.*, 1976
1973	FDA	South[3]	Farm and country elevator	146	99	1			Stoloff *et al.*, 1976
	University of Minnesota	Mexico	For human consumption	139[4]	96				Mirocha *et al.*, 1972

[1] ND = Not detected.

[2] Area where potential for Fusaria contamination was considered to be high or where Fusaria damage had been reported.

[3] Includes Southeast, Appalachia, Southeast MO, KY, TN, OK, TX, and CA.

[4] Levels in six positive samples were not reported.

Table 3: Survey of 1975 Wheat for Zearalenone

Levels of zearalenone (ppm)	Southeast	Western corn belt
ND[1]	35	58
<0.4	1	
0.4–0.9	2	
1.0–5.0	14	
5.0–10.0	1	
>10	1	
	54	58

[1] ND = Not detected.

Table 4: Coexistence of zearalenone with other mycotoxins

Commodity	Level zearalenone (ppm)	Mycotoxin	Level mycotoxin (ppm)	Reference
Corn	1.2	Aflatoxin	0.037	Shotwell *et al.*, 1970
Corn	0.6	Aflatoxin	<0.006	Shotwell *et al.*, 1971
Corn	14.5	Ochratoxin	3.1	Nowar, 1975
Corn[1]	ND-92	Aflatoxin	ND-1.7	Shotwell *et al.*, 1975
Corn[2]	0.2–10.4	Aflatoxin	0.1	Stoloff *et al.*, 1976
Barley	Not stated	T-2 toxin	Not stated	Formo, 1976

[1] Samples taken from various parts of bin and analyzed separately. Results are not representative of entire lot.

[2] Corn that was obviously mold damaged.

To evaluate the extent of the problem caused by any mycotoxin, one must have analytical methods available. Procedures for screening for, determining the quantities of, and confirming the presence of zearalenone have been published.

There are several multitoxin screening procedures that can be used to test simultaneously for zearalenone and one or more other mycotoxins in an agricultural commodity. Other mycotoxins (Table 5) are aflatoxin, ochratoxin, sterigmatocystin, patulin, citrinin, T-2 toxin, penitrem A, diacetoxyscirpenol, and penicillic acid. Steps in the procedures are extraction of the commodity, partial purification of the extract, and chromatography on thin-layer plates. Methods of purification have varied greatly from a dialysis membrane cleanup to column chromatography. Liquid-liquid transfers in separatory funnels and precipitations with ferric gel or cupric carbonate have also been used to remove impurities from extracts. The multitoxin screening methods have used TLC to detect zearalenone and other mycotoxins in extracts. A number of solvent systems have been reported.

Table 5. Multitoxin Screening Methods for Zearalenone and Other Mycotoxins

Other mycotoxins	Extraction solvent	Purification of extracts	TLC solvent-zearalenone	References
Aflatoxin, ochratoxin	Chloroform-water (250:25)	Silica gel chromatography	Ethanol-chloroform (5:95)	Eppley, 1968
Aflatoxin, ochratoxin, sterigmatocystin, patulin	Acetonitrile-4% potassium chloride (9:1)	Liquid-liquid transfer	Benzene-methanol-acetic acid (18:1:1) Hexane-acetone-acetic acid (18:2:1)	Stoloff *et al.*, 1971
Aflatoxins	Methanol-water (60:10)	Liquid-liquid transfer and cupric carbonate precipitation	Acetone-chloroform (4:96)	Thomas *et al.*, 1975
Aflatoxins, ochratoxins, sterigmatocystin	Chloroform-water (250:25)	TLC development benzene-hexane (3:1)	Toluene-ethyl acetate-90% formic acid (6:3:1) Benzene-acetic acid (9:1)	Hagan and Tietjen, 1975
Aflatoxins, ochratoxins, sterigmatocystin, patulin, citrinin, T-2 toxin, penitrem A, diacetoxyscirpenol	Acetonitrile-1% potassium chloride (9:1)	Membrane cleanup	Toluene-ethyl acetate-90% formic acid (6:3:1)	Roberts and Patterson, 1975
Aflatoxins, ochratoxin, T-2 toxin, diacetoxyscirpenol	Acetonitrile-4% potassium chloride (9:1)	Ferric gel precipitation	Toluene-ethyl acetate-acetone (3:2:1)	Stahr, H. M.
Aflatoxins	Methanol	Liquid-liquid transfer	Benzene-chloroform-acetone (45:40:15)	Seitz and Mohr, 1976
Aflatoxins, ochratoxins, citrinin, penicillic acid	0.5 N phosphoric acid-chloroform (1:9)	Column chromatography	Glacial acetic acid-benzene (1:9)	Wilson *et al.*, 1976

Two TLC schemes for differentiating and identifying a large number of mycotoxins including zearalenone have been reported; one for 18 mycotoxins by Scott *et al.* (1970) and the other for 37 mycotoxins and other metabolites by Ďuračková *et al.* (1976). How effective the Ďuračková method would be applied to the extracts of a number of different commodities and feeds containing low levels of mycotoxins has not been established.

In a study on zearalenone production by Fusaria species, Caldwell *et al.* (1970) extracted the molded corn with ethanol, dried the extract, and dissolved the residue in ether. The zearalenone solution was transferred into 0.25% sodium hydroxide, acidified, and re-extracted with ether to remove impurities. The residue from the final ether extract was taken up in methanol for TLC.

Using different solvents to avoid interfering substances and to increase the sensitivity, Mirocha *et al.* (1974) designed a similar extraction and purification method in which ethyl acetate was used to extract the commodity and chloroform was used instead of ether in solvent transfers. In the same publication, the detection and determination of zearalenone by TLC, ultraviolet spectrometry, and GLC of trimethylsilyl derivatives are reported. The dimethoxy and methyl oxime derivatives were prepared to confirm the presence of zearalenone. Analysis by GLC-MS and multiple ion detection were also described. The methods were applied by Mirocha *et al.* (1974) to a number of grains and feedstuffs (Table 1).

Earlier Vandenheuvel (1968) made an exhaustive study of the GLC of free zearalenone, its methyl oxime, and other related compounds and of their trimethylsilyl ethers.

A number of sprays have been used either to enhance the fluorescence of zearalenone on TLC plates or to form colored derivatives. An aluminum chloride spray was reported to enhance the fluorescence of zearalenone on TLC plates (Eppley *et al.*, 1974). Zearalenone on TLC plates turns brown when plates are sprayed with 50% sulfuric acid in methanol and heated at 120 C, but the reaction is hardly specific. It forms an intense blue spot after spraying first with potassium ferricyanide-ferric chloride and then with 2 N hydrochloric acid (Mirocha *et al.*, 1974). In 1974, Imere Sarudi jun reported using 4-methoxy-benzene-diazonium-fluoborate as a spray to form a colored azo derivative with zearalenone that was pale brick red. Spraying with a calcium hydroxide solution changed it to an intense yellowish red. After drying and spraying with ethanolic sulfuric acid, the spot became lilac colored. The series of sprays were reported to be very selective even in extracts of mixed feeds. Recently Malaiyandi *et at.* (1976) published on the use of a bis-diazotized benzidine spray that forms a brick-red derivative with zearalenone on TLC plates and is a more specific spray than potassium ferricyanide-ferric chloride.

Zearalenone has been confirmed on TLC plates by removing the fluorescent zone, eluting the zearalenone from the silica gel with methanol, and measuring the molar absorptivities (Stahr, 1975). The molar absorptivities in methanol are 29,700 at 236 nm, 13,909 at 274 nm, 6,020 at 316 nm. Stahr

measures the peak height at 274 nm to determine amounts of zearalenone. Brine shrimp (*Artemia salina* L.) is moderately sensitive to zearalenone (Harwig and Scott, 1971) and might be used to test eluates from TLC plates.

A collaborative study was conducted by Shotwell *et al.* (1976) to determine whether the method developed by Eppley (1968) for the screening of agricultural commodities for aflatoxin, zearalenone, and ochratoxin could be used to determine levels of zearalenone in white and yellow corn. The method, slightly modified had been applied to the screening for zearalenone in 567 corn samples collected by grain inspectors from commercial markets (Shotwell *et al.*, 1970; Shotwell *et al.*, 1971) and of 595 samples collected by the FDA from farms, elevators, and food processing plants (Eppley *et al.*, 1974; Stoloff *et al.*, 1976). Twenty-two collaborators from ten countries participated in the study. Average recoveries from spiked corn samples were 129% at 0.3 ppm, 101% at 1.0 ppm, and 88% at 2 ppm. The between laboratory coefficients of variation were 53% at 0.3 ppm, 38% at 1.0 ppm, and 27% at 2.0 ppm. The mean level of zearalenone in the five naturally contaminated samples analyzed ranged from 0.43 to 7.62 ppm. The mean coefficient of variation for all samples was 40.5%. The method was accepted in official first action by the Association of Official Analytical Chemists and the American Association of Cereal Chemists.

Collaborators used the following solvent systems with equal success: ethanol-chloroform (5:95), ethanol-chloroform (3.5:96.5), acetic acid-benzene (5:95), and acetic acid-benzene (10:90). The amounts of zearalenone on TLC plates were measured both visually and densitometrically. Jemmali (1974) reported the evaluation of zearalenone on TLC plates by reflectance fluorodensitometry. Known amounts of zearalenone were added to extracts of uncontaminated corn and chromatographed on TLC plates (Shotwell *et al.*, 1976). Amounts of zearalenone on developed TLC plates were determined by visual comparison with knowns and densitometrically. Excitation was measured at 313 nm and fluorescence at 443 nm. Results obtained densitometrically were more accurate than those obtained visually. The results obtained visually tended to be high.

In conclusion, when agricultural commodities are being analyzed for zearalenone, positive results should be confirmed particularly when the commodity has not been tested for zearalenone often. If amounts in extracts are determined by TLC, the levels should be checked by GLC and vice versa. If there is any question, a final confirmation should be made by mass spectroscopy.

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